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BARC

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PRESERVATION OF GERMPLASM

- WORKSHOP -

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Organizing Committee

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Preservation of Animal Germ Plasm - An Overview

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Efforts in germ plasm preservation have taken many diverse forms and directions over the years. Attention has been directed at numerous kinds of animals, from the largest whales, through the various classes of domesticated animals, down to the one-celled protozoa.

These efforts generally have followed two major courses of activity: (a) the natural propogation of whole organisms, or (b) the artificial manipulation of life cycles of either the whole organism or components thereof. Some of these efforts are summarized as follows:

I. Preservation by natural propogation

A. Farm and laboratory animals

1. Efforts in the United States

a. Jackson Laboratory at Bar Harbor, Maine, since its founding in 1929 has isolated, maintained, researched and disseminated laboratory mouse stocks around the world for use in research, teaching and product testing. Over 150 different mutant or inbred stocks are now on hand, plus numerous F_1 crosses. Various strains of rabbits also are being maintained for similar purposes.

b. In 1959, the AAAS sponsored the first formal symposium on "Germ Plasm Resources." It was organized by Dr. R. E. Hodgson, former Head of AHRD in ARS. A major aim was to chart a course for the protection and further improvement of plant and animal resources. However, to date, practically none of the proposals on protection have been implemented.

c. Dr. L. Sumption, at the University of Nebraska, developed a gene pool strain of 15 different swine breeds, starting in 1958. These included both popular American and "exotic" breeds. Two lines were derived from the strain in 1965 and selection experiments were initiated. At last report, one line is still being maintained. This is an example of preserving genes rather than separate breeds.

d. Dr. R. G. Somes, Jr., of the University of Connecticut, compiled the first extensive registry of chicken genetic stocks. The first edition was published in 1972. The second edition in 1975 also included information on turkeys and quail, as well as on Canadian chickens. The known ancestry, general description linkage groups, special traits and location of stocks were listed. These registries are recognized as models for this type of information.

e. The Animal Resources Branch of NIH supports efforts to preserve species around the world that are or may be useful in human biomedical research, such as non-human primates, miniature pigs and the armadillo.

f. No species of farm animals in the United States faces imminent extinction, but recent sharp declines in some breeds or strains are of concern to livestock leaders. An example is the rapid decline in certain strains of native ewes in western states.

2. Great Britain. Efforts have been made since about 1960 to preserve threatened breeds of farm livestock. In 1971 these efforts were consolidated into an organization called the "Rare Breeds Survival Trust." One of its major activities is to establish socalled "farm parks" where several threatened species are maintained and viewed by the public for a fee that helps support the Trust. It also provides information and sponsors research and educational projects on endangered breeds. It has been quite effective to date, but the future outlook is uncertain.

3. France. Of 22 formerly numerous local cattle breeds, all but 5 are now in danger of extinction. Sheep breeds are only slightly better off. Swine have suffered the most losses, with only two major breeds having any significant numbers. Thanks to fanciers, very few local breeds or strains of chickens have become extinct, but only a handful are economically important. However, France boasts of some 30 flourishing rabbit breeds. Interest in preservation efforts have greatly increased recently, but in the meantime, losses continue.

4. Other Countries. Various independent studies have been made, mainly on local conditions, but practically none have been followed up or implemented.

5. Agencies of the United Nations (U.N.). The U.N. Food and Agriculture Organization (FAO) first became interested in preserving germ plasm after World War II. In the 1950's, exploratory meetings were held to discuss possible approaches to the problem. Starting in 1966, international meetings were held annually on the general problems of preservation and on specific species.

   In 1972, the United Nations Environmental Program began working with FAO on preserving genetic resources. The two agencies then launched various cooperative pilot projects, including the following:

a. Prepared an inventory of breeds and strains in danger of extinction, including what was being done to preserve them.

b. Studied examples of breed dynamics on the Islands of Corsica and Sardinia.

c. Helped set up an agency to actively work to preserve the Kuri cattle of the Lake Chad area in Africa.

d. Established communication with various groups around the world working on the general problem.

e. Sponsored the first "World Congress on Genetics and Livestock" in Madrid in 1974. This featured a round table discussion on "Conservation of Animal Genetic Resources."

B. Wildlife. In general, the world's wildlife are now in a much more precarious position than domesticated animals, in terms of species survival. For example, in the United States and Puerto Rico alone, only two species of mammals and three of birds are thought to have become extinct from 1600 to 1850. But since 1850, 17 species of mammals, 28 of birds and 12 of fish are considered to have become extinct.

Efforts to protect endangered wildlife have taken many forms in recent years, including the prevention or limitation of hunting of certain species, the establishment of wildlife refuges, the prohibition of international or interstate trade in skins and animal products, and propogation of certain species in captivity for later return to the wild. However, the situation continues to deteriorate with each passing year for many species of wildlife.

II. Preservation by artificial manipulation of life cycles of either the whole organism or certain of its components.

A. Domestic livestock. The increase in artificial insemination after about 1930 paralleled improved techniques in semen preservation. The most dramatic results have occurred with cattle. Prior to 1950, only non-frozen semen was utilized, but Polge in the late 1940's discovered that glycerol afforded protection to spermatozoa during the stress of freezing to ultralow temperatures. His discovery is credited with opening the door for the remarkable subsequent development of cryobiological techniques for the long-term preservation of a variety of cell types. Most artificial breeding of cattle around the world now uses frozen semen. On the other hand, despite major research efforts with frozen semen in other species of farm animals, including poultry, progress has been slow and difficult.

B. Dogs. Artificial insemination with frozen semen has proven feasible.

C. Humans. Successful use of frozen semen has been reported.

D. Wildlife. Fresh semen has been used successfully for artificial insemination of several captive avian species. Use of frozen semen has not yet succeeded. Research efforts are continuing on freezing semen and other avian tissues for purposes of species preservation. Intensive efforts are also underway by the larger zoos, including the National Zoo, to obtain semen by electro-ejaculation methods from other captive wildlife such as the large cats, primates, certain ungulates and other rare species, for purposes of propogation in captivity.

E. Insects and other animals.

1. The nematode DD-136 with its associated bacteria is capable of attacking and killing many insect pests. Stock cultures of the nematode-bacteria complex can be preserved for long periods in specially prepared agar slants. For example, 27-year-old preparations are still intact here at Beltsville. Storage of various stages of the nematode in stock solutions or in glycerine suspensions for shorter periods of time is also feasible.

2. Insect tissues of various kinds have been preserved successfully in liquid nitrogen. These include blood cells, ovarian somatic tissue and embryos. To date, this method has been used mainly to study pathogens in insect pests, such as moths, butterflies and mosquitos.

3. Other insects in which preservation studies are being conducted for various purposes include the honey bee, coddling moth, gypsy moth, screw worm, corn borer and fruit fly.

4. Microorganisms such as protozoa have been maintained since antiquity for various purposes in extracts, suspensions, mashes and mixes, as well as in host animals and plants. Modern techniques such as freezing have allowed microbiologists to preserve intact organisms for extended periods and thereby avoid costly repetitious transfers in cultures. This also provides for better control and evaluation of experimental material.

F. Other work and future prospects.

1. Freeze-storing of embryos has proven feasible on an experimental basis. Research on embryo transplants also is making gains in that technique.

2. So-called genetic engineering methods may offer new challenges and opportunities for future control of germ plasm resources. Freezing somatic cells and transferring their nuclei into denucleated egg cells to produce clones of animals with identical genetic make-up is one possibility. Another is the irradiation or chemical manipulation of genetic material. However, these processes still are far from fruition and are probably beyond the scope of the present workshop.

The main reasons for preserving germ plasm can be divided into two main categories, as follows:

I. To assure survival of species or desirable breeds or strains facing extinction.

II. To allow more efficient control and manipulation of germ plasm when survival of a species, breed or strain is not a factor or of concern. This can be further subdivided as follows:

A. To facilitate efficient utilization of available genetic resources for improvement of economic traits.

B. To provide a stabilized population to serve as a control, bench mark or reference point for assessing treatment effects in experimental studies.

C. To allow, at lower cost than older methods, the more efficient control of life cycles in various organisms to be used for experimental and applied purposes.

General Problems and Ideas on Germ Plasm Preservation

Where survival of an endangered species is at stake, serious problems must be resolved before any efforts can succeed. Political decisions often are involved. This is especially true of wildlife that inhabit the oceans or several continents, for example, and may include such forms as the whale, polar bear and rare and exotic butterflies.

Formidable problems also are involved in preserving endangered domestic animals. For example, the ability or even the need to preserve all present strains or breeds is questioned. It has further been stated that "Preservation without utilization is doomed to failure." The main issues may reduce to: Who is to initiate any scheme of preservation? Who is to decide what to save? Who is to formulate the plans and direct the efforts? How long will the subject be preserved? Who will pay for it?

On the other hand, where manipulation, not extinction, of germ plasm is of concern, the critical need now appears to be further intensive research efforts in the appropriate disciplines of biology and technology. Different methods of preservation will, of course, need to be examined and perfected. However, most observers now believe that the primary emphasis of such research should be directly applicable to the science or field of cryobiology. Reliance on cryobiology now seems to offer the best and perhaps only immediate hope to successfully preserve animal germ plasm for most purposes.

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OVERVIEW OF PRESERVATION OF PLANT GERMPLASM

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The genetic vulnerability of crops has received considerable attention since the corn blight epidemics of 1970 and 1971. The corn blight epidemic was the result of lack of genetic diversity in the commercially grown cultivars. Germplasm is the foundation of variety improvement programs, and the conservation of genetic diverse germplasm is essential to those programs. Many international and national organizations and individuals have become involved in germplasm conservation. Emphasis has been given to plant explorations, and germplasm collections have increased. Procedures are being developed for coordinating the exchange, storage, documentation, evaluation, and replenishing of germplasm. Little emphasis has been given to the longtime preservation of germplasm.

The primary purpose for germplasm collections or repositories is to conserve genetic diversity for future plant breeding and improvement programs. Therefore, the objective should be to maintain the maximum genetic diversity in the preserved germplasm. The genetic diversity of preserved germplasm can be improved by the careful selection of new accessions and the reevaluation of accessions in existing collections.

The possible loss of native plant material in an area has been a major factor in determining plant explorations. The amount of genetic diversity of germplasm in an area also should be considered. The following may be used to indicate the genetic diversity of germplasm in an area:

1. Relation to the origin of the host.

2. The amount of intergression between plants.

3. The ecological and environmental variations.

4. The coexistence with important pests.

5. Genetic diversity of previously collected germplasm.

Special requirements should be established for the acceptance of germplasm from plant breeding and improvement programs. The recombination of more than one desirable characteristic in acceptable agronomic or horticultural plants should be considered as a prerequisite for new accessions.

The maximum genetic diversity in existing germplasm collections can be obtained by increasing the genetic diversity per accession or by increasing the number of accessions which can be preserved. The following may be used for improving the genetic diversity per accession:

1. The elimination of unique but undesirable mutations.

2. The elimination of duplicate accessions.

3. The development of composites and synthetics.

More efficient germplasm preservation methods must be developed to eliminate the loss of valuable germplasm. Additional germplasm is being obtained from plant explorations. The germplasm collected on these explorations must be introduced, stored, documented, evaluated, replenished, and then preserved. Not only introduced germplasm, but also commercial varieties of many crops are being lost. A recent study on the availability of old fruit varieties indicated that of 8,000 apple varieties known to exist about 70 years ago and the 900 named since 1920, only 1,500 are available today. More than 75 percent of the 400 strawberry varieties developed since 1920 have been lost.

The ability to store, document, evaluate, and replenish germplasm should be given careful consideration in planning the conservation of germplasm. Several million dollars are being requested to obtain storage and repository facilities for maintaining existing collections of crops. Procedures are being developed for documenting information available on accessions in existing collections. Additional documentation will be required as the collections increase in size and the evaluation programs are expanded. Many accessions in existing collections have not been evaluated because of insufficient staff and facilities. The cost of evaluating all of the accessions may be prohibitive. It has been estimated that the cost of growing and evaluating of one accession of corn will be $100 and there are 15,000 accessions in the CIMMYT corn collection in Mexico. The cost of evaluating perennial or tree crop accessions will be considerably higher. Accessions in collections are valuable only when they are used by other scientists. Material from collections furnished other scientists must be replenished. The replenishing of accessions will be very costly for some crops.

More efficient plant germplasm preservation methods would permit the maintenance of additional accessions in germplasm collections. Little research specifically on plant germplasm preservation has been conducted in the United States. The purpose of this workshop is to discuss research applicable to plant germplasm preservation.

Some of the methods used for preservation of microorganisms may be applicable to the preservation of higher plants. Viruses, bacteria, and fungi have been preserved in liquid nitrogen or other cryogenic materials. Vacuum drying and dehydration has been used effectively for preserving some microorganisms. The latter procedures require less equipment and little monitoring or maintenance.

Seed is the most common form in which germplasm of higher plants is preserved. The longevity of most seed in storage is correlated with low temperatures, humidity, and oxygen supply. The seed of some tree and tropical plants, which are referred to as recalcitrant seed, have limited longevity and cannot be stored at low temperatures or humidities. Frequent mutations and genetic abnormalities occur when the viability of seed in storage becomes less than 50 percent.

Germplasm of some very important crop plants cannot be preserved as seed because it is genetically unstable, the seeds contain disease producing agents, the plants do not produce seeds, or the plants are hybrids requiring vegetative propagation to preserve their identity. These crops must be preserved as pollen, shoot tip cultures, scions, cell cultures, or by other methods.

Germplasm of some crops is already being preserved as pollen. Most studies on pollen storage have been for only a relatively short duration. The reduction of temperature and humidity will prolong the viability of pollen of most crops. However, pollen of some crops such as Gramineae is recalcitrant and does not remain viable for a long period at low temperatures and humidities. Pollen of peach cultivars remained viable for 5 years at -20^{o}C. The preservation of pollen at temperatures of lower than -20^{o}C also has shown good results. The use of pollen for preserving germplasm greatly reduces the possibility of transmission of viruses and other disease agents. Light, temperature, growth regulators, and hormones have been used for controlling flowering of many crops. Flowering of potatoes is being induced by grafting potatoes onto tomatoes in some laboratories in Europe.

Shoot tip cultures are being used extensively for propagating plants and for eliminating viruses and other disease agents. The use of shoot tip cultures for longtime germplasm preservation has not been studied very extensively. The following are two examples in which shoot tips have been used successfully for longtime preservation:

1. In France, six replications of 800 grape cultivars have been preserved for 15 years as shoot tips in a 2 sq. meter area. To accomodate the same number of plants would require a hectare of land.

2. In California, strawberry cultivars have been maintained as shoot tips in culture for 5 years.

The successful preservation of grapes and strawberries as shoot tips suggests that germplasm of other crops can probably be preserved in culture rather than as plants grown in the greenhouse or field. The advantages of using shoot tips for germplasm preservation is that:

1. It is less costly because it requires less space and facilities.

2. Plants can be multiplied rapidly.

3. Shoot tips are less vulnerable to pests and environmental hazards because of the sterile conditions.

The use of scions and other plant parts also has been attempted for the preservation of a few trees and perennial crops. The preservation of woody plants as scions would greatly reduce the preservation costs for those crops. Most studies on preservation of scions have been limited to months rather than years. Pear scions have been preserved for over 2 years without losing their ability to develop shoots when grafted. Scions of apple, persimmon, and plum have been found to be viable after storage for more than 1 year. The application of additional methods and procedures for preparing the plant material and in adjusting the storage conditions may greatly improve the reliability and the length of storage of scions

Much research has been initiated on cell and tissue culture during the last few years. Techniques developed and information obtained from that research may be applicable to germplasm preservation. Cell and tissue cultures have been used for eliminating viruses and other disease agents from plants. Large plant populations can be maintained as individual cells. The research on cell culture has not been conducted for a sufficiently long time to evaluate its potential for germplasm preservation. A basic problem which is not solved is the differentiation of plants from single cells. This problem must be solved by additional research before cell cultures can be used for germplasm preservation.

In conclusion, the present methods for long time germplasm preservation are inadequate for maintaining the available germplasm, and they must be greatly improved to avoid the loss of genetic diverse germplasm being introduced from plant explorations and from plant breeding and improvement programs. New innovative methods for germplasm preservation are urgently needed and must be developed and utilized. Information on the use of pollen, shoot tip cultures, scions, and cell cultures for preserving germplasm should be investigated to determine what has been accomplished and how those methods may be utilized. The information presented and the discussions during this workshop should be helpful in identifying problems related to germplasm preservation and in determining what research should be initiated to improve plant germplasm preservation.

Research on Cryopreservation of Swine Spermatozoa

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For almost thirty years bull semen has been amenable to preservation by freezing. The use of the same freezing methods for boar semen resulted in recovery of some motility but complete loss of fertilizing capacity.

Sperm motility has been a useful criterion for assessing semen of many mammalian species but the initial post-thaw motility of boar spermatozoa has proven to be a notoriously poor indicator of fertilizing capacity. For this reason our initial research was directed toward finding laboratory criteria for assessing sperm damage during cooling and freezing.

We found by examining boar spermatozoa at high magnification with a phase-contrast microscope that the sperm acrosome (head cap) could be markedly altered by cooling. This acrosomal damage was not necessarily reflected in sperm motility. Therefore, acrosome morphology assessment has been subsequently used for the development of semen extenders, cooling, freezing and thawing procedures.

Semen Extender: Two extenders for freezing of boar semen have been developed at Beltsville. The first one, BF3 was composed of lactose, casein, Tris, citric acid, fructose and glycerol. The casein was effective in preventing acrosome damage during cooling and freezing. The second extender, BF5, was a modification of one developed at the University of Minnesota. It is composed of Glucose, Tes-N-Tris, Tris, Orvus ES Paste, glycerol and egg yolk. The Orvus ES Paste is a synthetic detergent that modifies the egg yolk lipoprotein. The BF5 extender is also quite effective in preventing acrosome damage during cooling and freezing.

Processing Procedure: The semen is collected into a vacuum bottle and allowed to gradually cool to 22 to 24° C over a 2-hr period. The semen is then centrifuged and the seminal plasma recovered. The sperm are resuspended in BF5 extender and cooled to 5° C over a 2-hr period. Additional BF5 containing 2% glycerol is added immediately before freezing.

Freezing: The semen is frozen on the surface of Dry Ice in pellets containing 0.2 to 0.25 ml each. A plywood board with 1/4" bolts placed at 1" intervals is used to make indentations in the surface of the Dry Ice block. The frozen pellets are then transferred to liquid nitrogen for storage.

Thawing: The pellets for one insemination are removed from the LN tank and scattered evenly in the bottom of an empty styrofoam shipping container and held for 3 minutes. Thereafter, the pellets are poured into a 400 ml glass beaker containing 40 to 50 ml of the thawing solution prewarmed to 50° C. The pellets are gently swirled in the beaker and thaw in under 20 seconds. The thawing solution (BTS) is composed of glucose, sodium citrate, sodium bicarbonate, EDTA and potassium chloride.

Current Research: At the present time research is directed toward (1) comparing various freezing techniques, (2) developing the technology to allow the storage of boar semen for up to 7 days in the unfrozen state, and (3) ascertaining the differences in the semen freezing ability among boars.

Immediate freezing on block of dry ice prior to LN storage

Changes in Acrosomal Enzyme Activity Associated with Cryopreservation of Swine Spermatozoa

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Successful cryopreservation of boar spermatozoa has only been a reality since 1971. Currently, commercial producers are using cryopreserved boar semen in ever increasing numbers. However, current procedures result in a less than optimum conception rate (55%) on the average. This less than optimum fertility results primarily from the damage sustained by the sperm cell during the freezing and thawing process. Apparent loss of viability in frozen boar sperm results from the fact that 35% of the sperm acrosomes show damage and approximately 60% are dead based on sperm motility measurements. Biochemical changes caused by freezing and thawing and their relationship to sperm viability is the subject of this paper.

The importance of the sperm cell acrosome and its contents to the fertilization process is well established. Acrosin, (EC 3.4.21.10) a proteolytic enzyme of the sperm acrosome, functions by digesting a pathway through the zona pellucida of mammalian ova, thus effecting sperm penetration into the ovum. Our studies to date indicate that acrosin is located in the inner and outer acrosomal membranes. In addition, it appears a slight amount of acrosin is present at the equatorial segment.

Boar testicular sperm contain only about one half the acrosin found in epididymal sperm. Acrosin activity of ejaculated boar sperm ranges from 8 to 35 mU/10^6 sperm, with an average of about 21 mU/10^6 sperm. An experiment was conducted in which semen was stored at 15°, 5° and -196° C in various extenders for up to 9 days to determine the relationship of sperm acrosin to morphological changes occuring in the sperm acrosome. Four different extenders were used. Egg yolk as an extender component enhanced acrosin activity by 23%. Over the 9 day storage period, acrosin activity decreased by 26%. However, the percentage of normal acrosomes decreased by 74% over the same time frame. There was no significant relationship between acrosin activity and percentages of normal acrosomes as determined by phase-contrast microscopy, indicating that acrosin activity is less susceptible to storage conditions and temperature than is the morphological integrity of the acrosome.

Loss of acrosin to the extracellular medium was determined in an experiment in which unextended sperm were frozen at -196° C either one or five successive times. Less than 1% of the acrosin of control sperm was lost to the plasma, as compared to 22% in the five time frozen sperm. Washing the sperm (part of the extraction procedure) resulted in losses of 11% in the control and 25% in the sperm frozen five times. Freezing boar semen in the BF-5 extender, according to the Beltsville freezing procedure results in significant protection for the sperm acrosin complement. Initial results employing an extraction procedure with incubation in both Hyamine and Triton X100 indicated that about 20%

of sperm acrosin activity was lost. A more sensitive extraction procedure more recently used indicates approximately 10% of the acrosin activity is lost during freezing and thawing. However, new evidence indicates that the quantity of acrosin lost may not be as important as what form acrosin is in when it reaches the site of fertilization.

Acrosin exists in up to five molecular forms in the boar sperm and our results show that two or three of the molecular forms are the result of autocatylytic breakdown of the zymogen, proacrosin. Further studies are underway to verify this and determine the effect of freezing and storage on proacrosin.

Current areas of research effort include. 1) Development of a freeze-damage assay. 2) Isolation of proacrosin and the role that freezing and thawing and uterine/oviductal environment play in the autocalytic process. 3) Relationship of boar seminal plasma inhibitor of acrosin to semen freezability and individual boar fertility. 4) Development of a more sensitive immunoelectrophoretic method of measuring acrosin/proacrosin.

Methodology in Freezing Poultry Semen

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Spermatozoa of the chicken and turkey are maintained in the oviduct for about 10 days after a single insemination with fresh semen and the subsequent eggs laid during this period are subsequently fertilized. It has not yet been possible to produce this pattern of fertility in hens using frozen-thawed semen. One major objective of this laboratory is to develop method(s) for preservation of chicken and turkey semen. The initial phase of our program dealt with a systematic evaluation of a standard freezing technique to determine the optimum dilution rate, cryoprotective agent, cooling and equilibration time to insure maximum recovery of the sperm's fertility capacity. Also, the relationship of various methods of insemination and the number of sperm/dose to the fertility of frozen semen were studied.

Semen was collected from mature males and subjected to the following experimental treatments in sequential order; (a) diluted 1 part semen with either 1, 3 or 5 parts Beltsville Poultry Semen Extender, (b) stored at +5°C. for 0.25, 1, 2, 4, 6 or 8 hrs. prior to the addition of 4% DMSO, (c) DMSO was added after cooling at a final concentration of 1 to 7% or (d) semen was equilibrated with 4% DMSO at +5°C. for the same periods used in b. In all experiments, the diluted semen was transferred after equilibration to glass ampules and cooled at a rate of 1.1°/min. from +5°C. to -20°C., placed for 3 min. in liquid nitrogen vapor, then stored at -196°C. for 48 hrs. Samples were then thawed in an ice bath and hens were inseminated weekly for 5 wks. with 50 million total sperm. Experiments were also designed to study the effect of varying the weekly insemination dose of frozen semen from 50 to 200 million sperm and the effectiveness of inseminating a set number of sperm in a bulk dose or in equal fractions. Eggs were collected daily, incubated at 7 day intervals, fertility determined by candling on Day 7 of incubation and expressed as the percent viable embryos. Unfrozen samples of the same semen pool were used as controls in all experiments.

Regardless of the prefreeze treatments employed, fertility was significantly lower in the frozen-thawed samples than the unfrozen controls. The highest level of fertility with frozen semen was achieved by diluting semen 1:5, adding 4% DMSO after 2 hrs. cooling at 5°C. in 8 equal fractions over a 2 hr. equilibration period and exposing the samples to liquid nitrogen vapor for less than 1 min. before storage.

Increasing the number of frozen-thawed sperm inseminated from 50 to 200 million resulted in a significant increase in fertility from 21 to 54%. Better recovery of fertilizing capacity was achieved by inseminating 150 million sperm in two equal fractions as opposed to inseminating the total number in bulk.

Results from these studies indicate that the prefreeze conditioning of the sperm and the mode of insemination were important factors in the recovery of the fertilizing capacity of frozen-thawed semen. Also, the freezing procedure developed in this study appears promising because a sufficient number of spermatozoa remained viable as evident by the fertility levels (>50%) obtained with thawed semen samples.

Possible Applications of Germ Plasm Preservation in Cattle

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When germ plasm preservation is viewed as long-term preservation of genetic material, it can be stated that little or no such efforts are being made in cattle. A small-scale start has been made in England to maintain a few herds of rare breeds of cattle (Rare Breeds Survival Trust). In many countries, frozen semen banks are maintained, but these are only used for short-term on-going genetic improvement, rather than for long-term conservation.

Frozen semen provides a means of storing haploid genetic material for very long periods, although our experience at present does not exceed 25 years. There is some indication of decreases in fertilization capacity over time. Some progress has been made in freezing embryos. In 1973, the first calf was born after transplanting an embryo frozen (-196°C) in the blastocyst stage. This follows extensive successful freezing of rabbit embryos. Banking frozen embryos would in some respects be preferable to frozen gametes, although costs of implanting embryos may require further reduction to be more cost effective. If it were possible to clone embryos before freezing, this would be even more advantageous.

The possible usefulness of germ plasm preservation depends largely on the probability of genetic calamities occurring, which cannot be evaluated very precisely. Some of the possible applications are: (1) to introduce genetic diversity not currently present in a commercial population; this includes the introduction of specific genes, such as those that confer disease or parasite resistance. In addition, blocks of genes affecting quantitative characters and performance may be needed, such as when genetic plateaus to selection are reached, when selection goals have to be modified, or when a new breed needs to be introduced into a habitat. (2) To use the preserved genetic material as a control in genetic selection experiments (this is currently being done in USDA dairy cattle experiments at Beltsville and Lewisburg, Tennessee). (3) To capitalize on favorable interactions; if favorable genotype x genotype interactions (sire x maternal grandsire) are detected, frozen semen could be preserved to capitalize on these for an extended period. Likewise, if desirable sire x environment interactions are found, semen could be stored to utilize these combinations for a long period. (4) For uses in physiological research; certain types of genetic variants may be very useful in investigations of various physiological phenomena and processes. Examples of this would be as genetic models of disease, providing optimal experimental organisms (such as those having insensitivity to a particular hormone), or providing animals that possess maximum responsiveness for bioassay purposes.

Unfortunately, it is virtually impossible to predict the future value of germ plasm preservation in cattle. This really depends on the chances of events such as the appearance of virulent new pathogenic disease organisms, the occurrence of pleateaus to selection progress due to diminished genetic variation or other reasons, or the need to radically alter selection goals due to changes in consumer preferences, conditions of production, etc. Germ plasm conservation may be likened to a form of insurance, since its use only comes into play in the event of catastrophic events. The penalties for failure to take out insurance would be very great should one of these unlikely events occur.

Cryopreservation of Protozoan and Helminthic Germplasm

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The major reasons for preserving germ plasm of parasites at the Animal Parasitology Institute are:

1. Long-term preservation of reference strains and isolates
2. Avoidance of serial animal passages
3. Accumulation and storage of materials when available for later experimental use.

The first reason is exemplified by the work of Doran and Vetterling with oocysts of Eimeria spp., that of Isenstein and Herlich with Trichostrongylus spp., that of Barry and Ann Chute with Histomonas meleagridis, and our own storage of stabilates of Babesia spp. Storage of Babesia spp. helps to avoid the very real problem of antigenic drift and allows us to work with parasites of a given serologic specificity for long periods of time. Dar et al. used cryopreservation to preserve metacyclic and blood stream forms of various species of Trypanosomes and found that antigenic variation was completely avoided by this technique.

Those of us who work with parasites of large animals greatly appreciate the fact that we do not have to spend $50 to $200 per month for passage animals to maintain each parasite. Aside from the monetary expense for animals, continuous passage requires expensive technical assistance, and also has the drawbacks of possible contamination or loss of the agent.

The third reason is concerned not only with parasite preservation but also with storage of host or parasite tissues for determination of enzyme activities, drug levels, and detection of metabolic products.

In general, cryopreservation of protozoa involves the use of cryoprotective agents, especially glycerol and dimethyl sulfoxide. Glycerol (5 - 15%) is usually mixed with blood containing parasites or with parasites suspended in a buffer or tissue culture medium and allowed to equilibrate for 30 minutes to several hours at room temperature or at 4°C. DMSO (10%) has the advantage that equilibration is almost instantaneous and freezing can be done almost immediately.

In some cases, it is vital that cooling be done at a controlled rate of 1-2°C/minute in the range from 20°C to -25°C. Cooling faster than this results in lowered viability of many protozoa. Fortunately, in the case of Babesia spp., we found that parasitized blood could be frozen rapidly by "shelling" the

vials in a dry ice-acetone bath and then plunging the vials into liquid nitrogen, or even by plunging vials directly into liquid nitrogen. Babesia equi treated in the latter manner was still viable after 8 years of storage at -196 C.

Rate of thawing also is important for preserving viability of protozoa. In most cases rapid thawing is recommended, i.e., use 37° - 43° waterbath for thawing.

The following are examples of work in this area performed at the Animal Parasitology Institute:

Doran and Vetterling froze sporozoites of Eimeria meleagrimitis and E. tenella using 7% DMSO and controlled freezing (1 C/min) Doran reported that freezing of Eimeria sporozoites did not affect their ability to grow in cell culture.

Chute and Chute determined that Histomonas meleagridis could be stored for at least 345 days in a menstruum of 84% Medium 199 (Hank's BSS), 8% chicken serum and 8% DMSA with cooling at 1°C/min down to -30°C and 10°C/min to -196°C.

McLoughlin and Chute successfully stored Trichomonads from cattle and chickens for at least 10 years without any change in resistance markers of various strains. They also stored stages of Leukocytozoan spp. in their insect vectors for limited periods.

Isenstein and Herlich reported that Trichostrongulus axei could be frozen in distilled water (no cryoprotective agent) and maintained in liquid nitrogen vapor (-170°C) for 80 days. T. colubriformis could be stored for at least 38 days.

We have used freezing to preserve parasites or tissue from hosts to which parasiticides have been administered to study levels of drug in tissues. This procedure has also been used to preserve parasites in order to accumulate sufficient numbers so that studies of intermediary metabolism can be done.

Frerichs et al. reported the following data for longevity of storage of various species of Babesia:

B. caballi	1040 days	B. canis	381 days
B. equi	8 years	B. rodhaini	328 days
		B. microti	159 days

Cold-hardiness of nematodes. I. Effects of rapid freezing on the eggs and larvae of Meloidogyne incognita and M. hapla.

Richard M. Sayre, Nematology Laboratory, PPI

Meloidogyne hapla, a species of root-knot nematode that can survive freezing temperatures, and M. incognita a species that cannot, were observed while being frozen on a microscope stage where temperatures could be lowered to $-40^{\circ} \pm 0.1^{\circ}C$ at a maximum rate of $10^{\circ}C$/min. Eggs and larvae survived for short periods in supercooled water and in ice, but formation of internal ice was lethal to both species. At a lower rate of cooling, $1^{\circ}C$/min., there was no difference in the ability of the species to survive subzero temperatures. However, when larvae were frozen in physiological salt solution or egg-masses were stored for long periods at temperatures slightly above freezing and then frozen, significantly more M. hapla survived than did M. incognita.

Freezing and storing Ditylenchus dipsaci in liquid nitrogen.

Richard M. Sayre and Shuh-wei Hwang, Nematology Laboratory, PPI, and 13208 Georgia Avenue, Silver Spring, Maryland 20906.

After 18 months of storage at -150 C, some larvae of Ditylenchus dipsaci, which had been treated in a 7.5% solution of dimethyl sulphoxide and cooled to -25 C before storage, were still viable on thawing. Some survivors penetrated and developed normally in stems of alfalfa seedlings. Tests showed that active larvae could be frozen directly, thus eliminating the need to use the quiescent stage of this nematode previously thought necessary for successful storage at cryogenic temperatures. The method described is suitable for long-term storage of D. dipsaci and may, with slight modifications, be used to preserve other plant-parasitic nematodes.

Freezing and storing a tardigrade (Hypisibius myrops) in liquid nitrogen.

Richard M. Sayre and Shuh-wei Hwang, Nematology Laboratory, PPI, and 13208 Georgia Avenue, Silver Spring, Maryland 20906.

Previous reports on survival of tardigrades at cryogenic temperatures involved partially dehydrated individuals taken from their natural habitats. In our tests active specimens of Hypisibius myrops were reared in culture dishes using methods developed by Sayre [Trans. Amer. Microsc. Soc. 88:266-274 (1969)]. Larval and adult forms extracted from cultures were resuspended in 7.5% (v/v) DMSO. Aliquots of 0.5 ml containing about 1500 tardigrades were pipetted into 1.5 ml amouples which were subjected to 2 cooling rates. Group A (20 ampoules/group) was placed in a BF-4-1 controlled rate freezer at -28°C and held between -28 to -32°C for 25 minutes. Group B was placed in the freezer at +20°C and cooled at the rate of 1°C/min to -28°C taking approximately 48 minutes. Immediately after the treatment of each group, the temperature was then quickly lowered to -196°C, and both groups were stored in liquid nitrogen for one month. Ampoules were thawed in a water bath at 22°C. The survival rate for tardigrades from group A was 45.0 $\pm$ 8.0% ($P<0.05$), while only 17.0 $\pm$ 4.3% ($P<0.05$) of group B survived. The more rapid cooling rate was significantly better. Surviving tardigrades returned to culture dishes moved and fed normally. Some incidences of moulting and encystment were recorded over a period of 24 days. No viable tardigrades were found after that time. Normally this tardigrade completes its life cycle in approximately 17 days at 25°C. Results indicate that freezing injury was not repairable under our usual rearing procedures and a more favorable environment is needed or the freezing method must be modified to reduce injury.

Preservation of Living Infective Stage Larvae of the DD-136 Insect Parasitic Nematode, Neoaplectana dutkyi Jackson, and Cultures of its Associated Insect Pathogenic Bacterium.

S. R. Dutky
Insect Physiology Laboratory
Plant Protection Institute

ABSTRACT

Living infective stage larvae of the DD-136 nematode have been sustained at the Beltsville laboratory from the time of its first recovery in September 1954 to the present time. These are stored in sealed oxygen filled containers or in cotton stoppered flasks at 7 C. The storage fluid presently used is formaldehyde solution USP 1 + 1000 H_2O made 0.02 molar in respect to sodium hydroxide. The addition of sodium hydroxide has prevented overgrowth by Aspergillus flavus, a frequent fungus contaminant in the Galleria mellonella larvae used as the rearing host.

Monoxenic cultures prepared by combining surface sterilized embryonated ova of the nematode (treated with 0.1% NaOCl aqueous solution for 15 minutes) with a pure culture of the associated bacterium tested for good insect pathogenicity, strong antibiotic production and good production of the characteristic red UV fluorescent pigment. The substrate used for growth and development of the nematode is peptone-glucose agar slants to which an autoclaved cube of pork kidney is added. These slant cultures can be held for 2 months at 25 C, and then should be transplanted to fresh medium. Addition aseptically of 1 ml of sterile water monthly can sustain these cultures for a much longer period (up to 6 months).

Viable cultures of the DD-136 nematode associated bacterium have been preserved on oil flooded slants of tryptose-phosphate broth agar since 1957, and cultures of nematode associated bacteria from other nematode species have been preserved from 1949.

Oil-flooded peptone-glucose agar cultures of entomogenous fungi Entomophthora apiculata and Entomophthora coronata have been maintained since 1965 and still retain their sporulating characteristics and insect pathogenicity.

Problems in the Preservation of Germplasm at the Cell and Tissue Level

Gideon W. Schaeffer
Cell Culture and Nitrogen Fixation Laboratory

The rapid progress in the past 25 years in molecular genetics is based in part on the availability of large numbers of microbes with known physical and biochemical characteristics genetically prescribed. Techniques are now being developed with large numbers of plant cells to identify, study, and prescribe genetically useful biochemical systems. Large numbers of somatic cells from single plants may now be obtained from isolated protoplasts originating from leaf, stem, and root tissue, from amorphous callus or from cells in liquid suspension. Other sources of large numbers of cells include the pollen microspores from anthers, and meiotic cells from developing eggs or embryonic tissues. The uses for large numbers of cells from a single plant and protoplasts from these cells along with concepts pertaining to their preservation will be briefly mentioned.

Somatic hybridization, genetic modification and biochemical cell selection from somatic and gametic tissues, particularly the pollen microspores, are techniques for obtaining cells prescribed genetically. The recovery of altered or selected cells as callus or cell aggregates allows preservation of the new genotypes. Subsequent recovery of the genotype as a whole plant permits the preservation of the selected cell as seed material. However, the differentiation and morphogenesis of tissue culture into intact plants cannot now be done systematically with most crop and horticultural plants. Thus, plant preservation techniques are required for all stages of the cell culture-modification-recovery process. Newly selected and identified cell types cannot now be adequately and reliably preserved without extensive research at several stages of this multiphasic process.

Tissue Culture -- An Easy Method to Obtain Male-Sterile Plants

Patricia Sarvella, Gideon W. Schaeffer, and H. A. Skoog
Cell Culture and Nitrogen Fixation Laboratory and Tobacco Laboratory

Male-sterile plants must be obtained by means of crossing male-sterile and normal plants. In some crops this requires considerable effort. Plant tissue culture techniques might provide an easy way to preserve valuable germplasm. A cytoplasmic male-sterile Nicotiana tabacum line was selected for culture of the anthers. Many plantlets were obtained from the cultures, i.e. one anther gave at least 75 plantlets. About half of the plants were male-sterile diploids. The remainder were haploids which could easily be doubled with colchicine. The next generation from the diploid plants on tissue culture again gave male-sterile plants. Thus, use of cytoplasmic male-sterile pollen was capable of regenerating male-sterile plants.

RESEARCH ON PRESERVATION OF SEED VIABILITY AT BELTSVILLE

A. Abdul-Baki, Seed Research Laboratory

Early research on seed preservation and viability was conducted at various laboratories in the Division of Seed Investigations. The creation of a Seed Research Laboratory within the Agricultural Marketing Division which recently became the Agricultural Marketing Research Institute, led to the consolidation and strengthening of seed research and gave birth to some strong continuous programs on seeds particularly in the areas of viability, vigor and deterioration.

So far, research on preservation of seed viability at Beltsville focused on establishing optimal external environmental requirements for prolonged storage. It focused on the effects of seed moisture, storage temperature, gas composition of the container, and container type. It also examined the effect of prestorage operations such as harvesting, drying, processing, shipping and chemical treatments on storage life of grass, field crop and vegetable seeds.

Studies on preserving seed viability in storage were limited to experiments in which storage temperatures ranged from room temperature to deep freeze temperatures (about -18°C), and from high seed moisture levels to low ones (20% to 4% moisture). The results, in some cases, established new original information and in others confirmed and extended observations that had been published by other researchers outside Beltsville. These findings led to many important conclusions such as: (a) Dormancy is best maintained at low temperatures and can be lost in a short time at room temperature; (b) High seed moisture (above 14%) induces rapid deterioration and very low seed moisture (below 6-7%) increases the frequency of hardseededness; (c) Prestorage operations, such as harvesting, drying, milling and cleaning, affect storability; (d) Deteriorative processes are specific and once they take place they are irreversible, (e) Vigor is lost before viability; (f) the frequency of genetic abnormalities increases in seeds during storage and the largest increase occurs under unfavorable storage conditions of high temperature and moisture; (g) Least genetic changes are noticed when seeds are stored fully imbibed.

Research on maintaining seed viability over long periods of time should utilize embryos rather than whole seeds. Some of the already developed cryogenic techniques should be evaluated particularly with regard to the effect of very low temperatures on viability and frequency of genetic changes. Long term studies should explore the relationship of seed longevity on one hand and the stability of DNA and the ability of the species to maintain and repair nuclear damage.

Preservation of morphological structure and viability of plant tissues frozen in the presence of cryoprotective agents and the freeze preservation of viruses and viroids as purified nucleic acid.

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The techniques of freeze-drying, freeze-fracturing, and freeze-etching used to prepare specimens for electron microscopy are useful for morphological and cytological studies. One advantage of these approaches is that the biological material can be prepared without the need for chemical fixation. One can hope that the pictures obtained show the appearance of "living" material. Unfortunately, too little effort has been expended to demonstrate that the samples are actually in the living state at the time replicas are made. A few examples have been presented to demonstrate survival of cells under the conditions of freezing and thawing used. These have been done with rather tolerant organisms, such as yeast and bacteria. Even with such specimens it has been demonstrated that those which look the best in the electron microscope do not have the best survival. Survival of some plant cells frozen in the presence of dimethyl sulfoxide at low concentrations has been demonstrated.

We have recently discovered that unfixed plant cells frozen in mixtures of sucrose and glycerol in phosphate buffer provide conditions desirable for high resolution freeze-fracture specimens. Preliminary studies suggest that these mixtures, or these in combination with other cryoprotective agents, will permit survival of plant cells or tissues as well as providing the conditions necessary for high-resolution electron microscopy of "living" specimens. We are currently studying these possibilities. It is presumed that slight modifications of such procedures would be useful as well as for both the preservation and electron microscopy of unfixed microbial and animal specimens.

Infectious nucleic acid has been obtained by the degradation of a number of viruses. The purified nucleic acid from these and purified viroid nucleic acids can be maintained in the frozen state.

Use of PVI

Proposed Preservation of Sugarcane and Sweet Sorghum Germplasm

A. G. Gillaspie, Jr. and R. G. Mock

Applied Plant Pathology Laboratory

The World Collection of sugarcane germplasm is currently maintained vegetatively by the ARS in Beltsville and at Canal Point, Florida. This collection contains several thousand clones and it is expensive to maintain. A more economical method for preservation of the germplasm would be worthwhile. Sugarcane can be cultured in vitro and one line of investigation should be liquid nitrogen preservation of cells and tissues in culture. Carrot, belladonna, and sycamore have been preserved for up to 10 months with no changes in cell morphology or embryo-genic potential. Liquid nitrogen freezing of shoot apices with the carnation is another possible approach to preservation, but thus far carnation apices have been stored only 2 months. Cryoprotectants such as glycerol and dimethyl sulfoxide have proven effective for these systems. The rate of freezing and of thawing was important. The cell suspensions were viable only after slow freezing and rapid thawing while the shoot apices were viable after rapid freezing and thawing.

All of the breeding work for sweet sorghum in ARS is done at Beltsville in the greenhouse. It has been difficult to coordinate the flowering of male and female parents and it is difficult to obtain satisfactory pollen. Thus, a method for preservation of pollen would be advantageous. Pollen has been stored by vacuum-drying, freeze-drying, freezing, and organic solvents. The important factors in freeze-drying is the rate of freezing, the conditions during storage, and the rate of rehydration. For example, with corn pollen rapid freezing, drying at -60° C for 75 hrs., storing in vacuum or nitrogen at 0-5° C, rehydrating at 5° C at 10 percent relative humidity yielded viable pollen after 130 days in storage. For freezing in liquid nitrogen, rapid freezing and thawing is usually best. Polyvinyl-pyrrolidone powder has been used as a cryoprotectant for freezing at -20° C with grass pollen. The organic solvent method uses pollen dried at -10° C over silica gel which is then soaked in acetone, alcohol, ether, chloroform, and other organic solvents at 4° C (lilly pollen was viable after 80 days). We plan to investigate freezing with and without cryoprotectants and freeze-drying as methods for sweet sorghum pollen storage since both corn and sugarcane pollen have been successfully stored in these ways.

In order to assay the techniques a useable method for germinating the pollen is needed. Several media have been reported for sugarcane pollen germination, but none of these have proven adequate for sweet sorghum pollen in our tests. These media contain sucrose and some also contain boric acid, magnesium sulfate, and potassium nitrate.

Preservation of strawberry cultivars in vitro

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Brooks and Olmo in the Register of New Fruits list almost 400 strawberry cultivars introduced since 1920. At least three quarters of these have been discarded and lost over the years. Some of them carried genetic characteristics which would be needed today. In the proposed germplasm depository the preservation of cultivars is planned by growing them in pots in the greenhouse. This requires special care. Mite infestation, nematodes, and rooting of unintentional runners in adjacent pots are the potential problems.

Excision and rooting of tips, in conjunction with thermotherapy, has been used at Beltsville since 1963 to eradicate viruses. During the past 13 years some 2500 cultures were made and 500 virus-free plants were produced. Mullin and Schlegel used the tip culture to produce plants and store them in the cold. The oldest plants in storage are about 5 years old.[1/] The techniques and medium used for these studies are well established.[2/] At the present we have 110 plants of 37 cultivars stored here in the Fruit Laboratory for up to 6 months. The plants are stored at +1°C and the storage appears satisfactory. This opens a wide range of possibilities to store plant tissues in cold and further research is necessary to develop the methods for other plants as well.

1/ Mullin, R. H. and D. E. Schlegel. 1976. Cold storage maintenance of strawberry meristem plantlets. HortScience 11:100-101.

2/ Galzy, R. 1970. Recherches sur la croissance de la vigue saine et court-nouée cultivée "in vitro". Thesis. Univ. of Clermont, France.

Abstract

Exchange and Conservation of Germplasm in Tissue Culture.
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Exchange of Germplasm

Tissue culture techniques, particularly aseptic culture of plantlets, has been utilized as a safeguard in the international transfer of plant genetic stocks. The objective is to facilitate the exchange of plant genes while lowering the chances of inadvertently exchanging hazardous pests and pathogens. The risk is lowered in two ways. Firstly, the size of a consignment is vastly reduced. An imported variety or clone may be represented by meristem tips or excised buds or embryos instead of cuttings, scions, tubers, seeds, etc. Secondly, the aseptic plantlet system has built-in pest and pathogen detection capability. Cultures which remain uncontaminated with fungi or bacteria must be free of all pests and pathogens except obligate parasites (such as rust fungi or viruses) and certain fastidious organisms. The technique has been used to transfer sugarcane, potato, asparagus, and other temperate and tropical plant genera from the USDA Plant Quarantine Facility, Glenn Dale, Maryland to (1) the quarantine station in Nairobi, Kenya, and (2) the Potato Improvement Center, Lima, Peru. In addition, plantlets have been transferred from a research station in Taiwan via the University of California at Riverside to the Glenn Dale Station. Tissue culture techniques may also be incorporated in pest and pathogen eradication procedures as well as for the long term storage of imported genetic stocks to prevent contamination by domestic pests and pathogens.

Problems in the Propagation of Cymbidium Mosaic Virus-free Cattleya Orchids in Tissue Culture

R. H. Lawson and S. S. Hearon
Ornamentals Laboratory, PGGI

Production of virus-free plants has often been successful when small shoot tips or explants are grown on a nutrient agar or liquid culture medium. Many Cattleya orchids propagated from cymbidium mosaic virus-infected plants in tissue culture remain virus-infected when conventional methods of propagation are employed. The conventional method includes excision of large tissue pieces, usually 1.5 to 2 mm, from the vegetative shoot.

Extracts were made from exposed shoot tips measuring about 1.0 mm that included the apical dome and two surrounding leaf scales. Virions were detected in the electron microscope in 80% of the tips tested. In thin sections of embedded shoot tips, virus particles were observed in cells of the two innermost leaf scales and in cells of tissues below the point of attachment of the outer leaf scales that were removed. Groups of infected cells were surrounded by apparently noninfected cells. Virions were in parenchyma and epidermal cells scattered through the cytoplasm as loosely packed aggregates.

Meristem tissue pieces, including only the meristematic dome, showed little survival when explants less than 0.5 mm were cultured on a synthetic medium (M. Ishii, Acta Horticulturae 36:229, 1974). Many tips 0.5 to 1.0 mm survived in culture, but were virus-infected. Tissue pieces 0.2 to 0.5 mm cultured on callus tissue of ginger or Cattleya had a low percentage survival, but some were free of cymbidium mosaic virus.

Tissue 1 x 2 mm or [illegible] readily on an agar medi

The Importance of Cryogenic Preservation of Germplasm in Cultivated Mushroom Production and Research.

James P. San Antonio
Vegetable Laboratory

ABSTRACT

Cryogenic preservation of stock cultures of the cultivated mushroom, Agaricus bisporus (Lange) Sing. has several important advantages for mushroom production and research. Preservation of the actual mycelium-colonized grains "spawn" used commercially as the planting inoculum has been demonstrated to be a successful and convenient method for maintaining master cultures. Results to date indicate that there is no significant difference in the quality or quantity of mushrooms produced from mushroom cultures preserved at - 160 to - 196 C. Commercial manufacture of spawn of the cultivated mushroom is as much if not more an art as the actual growing of mushrooms. Because of liquid nitrogen preservation of spawn stocks of the cultivated mushroom, it is now possible to begin rational spawn testing programs. Cryogenic preservation permits convenient and reliable storage of special stocks and a large number of different mushroom isolation useful for breeding.

PRESERVATION OF RUST SPORES IN LIQUID NITROGEN

R. A. Kilpatrick and D. L. Harmon, Germplasm Resources Laboratory

Rusts of wheat and oats, caused by Puccinia spp., are often major diseases where the crops are grown. The heterogeneity of biotypes within rusts is known to change frequently. The use of resistant cultivars is the only economical means of preventing epidemics like those occurring in 1950 and in previous years. Greenhouse screening provides rapid information on disease reaction of seedlings.

In 1960, urediospores of two cultures (56-51A and 15B-51A) of the stem rust organism, P. graminis tritici, were collected for a long term storage test. Spores were subjected to four treatments as follows: 1) vaccuum dried and stored at 4 C°; 2) vaccuum dried and stored in liquid nitrogen (LN); 3) air dried for 24 hrs and then stored in LN; and 4) hydrated for 4 hrs and then stored in LN. Each vial (5 mm in diam.) contained approximately 10 mg of spores, sealed with a double burner, oxygen blow torch and then placed directly in LN. Vials were removed after 6 months storage and annually for 5 yrs, and then at 7 and 10 yrs storage to determine germinability and infectivity.

Germination of urediospores in treatment No. 1 showed a sharp decline in viability after 5 yrs storage. Only traces of germinating spores were observed between the 5 and 10 yr test. After 10 yrs, germination of vaccuum dried spores (No. 2) was better than hydrated spores (No. 4). Germination of air dried spores (No. 3) of both cultures was essentially the same as that recorded after 6 months. Urediospore germination was, in general, higher in culture 56-51A than those in 15B-51A.

Data on infectivity demonstrated viability. Symptoms were more prevalent on hypodermic-infected plants than on talc-inoculated seedlings. The advantages of using two criteria for evaluation of results was demonstrated. Germination tests are necessary but insufficient and misleading for determining infectivity.

The results have shown that rust spores can be air dried and stored in LN for over 10 years with little or no loss in germination and infectivity. Spores of crown rust (P. coronata avenae), collected in Puerto Rico, have shown contamination problems. A Helminthosporium leaf spot occurs in Puerto Rico and the fungus sporulates during the period of rust development. Harvesting spores from rust and Helminthosporium infected plants results in spore mixture. The use of contaminated spores in inoculation causes poor rust infection due to necrotic lesions and a toxin which are produced by the Helminthosporium fungus.

Storage of rust spores in LN provides a ready source of inoculum for use in genetic tests, screening of seedlings for resistance, or comparison of rust cultures from different years.

INSECT SURVIVAL IN THE COLD

Dora K. Hayes

Chemical & Biophysical Control Lab

Agricultural Environmental Quality Institute

ABSTRACT

Sullivan has demonstrated that the eggs of Eastern tent caterpillar, Malacosma americana, survived temperatures well below 0°C and at an altitude of 40,000 ft. He further showed that fractions of small populations of the adult American dog tick, Dermacentor variabilis, and the adult confused flour beetle, Tribolium confusum, survived 1 hr at -10°C. Pickens has shown that the parasitic wasp, Pachycrepoideus vindamiae, presumably in diapause, survived outdoor temperatures fluctuating above and below 0°C for at least 4 months. Hayes, Cawley, Schechter and Sullivan demonstrated that larvae of the European corn borer, Ostrinia nubilalis, conditioned under natural "short day", LD 13:11, in the fall enter diapause and resist cold under outdoor conditions at Beltsville for 5-6 months. Pupae of some species of Lepidoptera can be maintained for spans of at least 3 months at temperatures between 12-14°C.

Preservation of Insect Cells in Liquid Nitrogen

James L. Vaughn
Insect Pathology Laboratory

The preservation of insect cell lines is accomplished following well established procedures developed for the storage of cell lines from other animals. The procedure most frequently used for long term (12 months or longer) storage is in liquid nitrogen at -196°C or in liquid nitrogen vapor at -170° to -180°C. This storage is used to (1) reduce the work load of weekly transfers of unused cell lines, (2) prevent loss of valuable cell lines as a result of a laboratory accident, and (3) prevent the loss of or change in desirable characteristics of the cell lines. Such changes may be in the karyotype of the cells or in somatic characteristics such as antigenicity, growth rate or virus susceptibility.

The cells to be preserved are harvested from a vigorously growing culture by scraping with a rubber policeman and suspending in the growth medium. The cell concentration is adjusted to the desired level and a cryoprotective agent added. The most commonly used agents are glycerol and DMSO. In addition the serum level is increased to 14% to provide additional protection. The cell suspension is dispersed in 1 ml aliquotes to borosilicate glass ampoules which are heat-sealed.

The cell suspensions are frozen in a liquid nitrogen-cooled freezer in which the cooling rate can be closely regulated by controlling the flow of liquid nitrogen. The cell suspension is cooled at a rate of 1-2° per minute to a temperature of -60 to -70°C. The ampoules are then transferred to liquid nitrogen refrigerators and stored submerged in the liquid nitrogen.

The cells are recovered by removing the ampoule and thawing the contents rapidly in a water bath at 35° to 40°C. The contents of the ampoule are diluted with growth medium and transferred to a culture vessel. The viability of the recovered cells, as measured by a trypan blue dye exclusion test, is between 70-90% and normal growth is obtained after the first transfer. Cell lines have been maintained in this storage for 8 years.

DMSO will cause a [illegible] when added to the culture media. This could kill cells. Both the DMSO and culture media should be cooled to ice water temp.

Preservation of Rice and Forage Crop Germplasm

A. J. Oakes

Germplasm Resources Laboratory

The expense and service-type connotation associated with the preservation of forage and field crop germplasm over the years has relegated this essential task to a low priority among administrators and research scientists alike. This situation is unfortunate and requires remedial action. The sustained maintenance of germplasm containing maximum genetic variability is essential for the further development, improvement, and reduction of genetic vulnerability of forage and field crops. Long-term availability of such genetic building blocks is vital to the future of American agriculture.

It is important to preserve germplasm of those species and clones of forage crops which are propagated entirely or in part by vegetative means. The work and money spent in the sustained maintenance of forage and field crop germplasm compensates, in part, for continuous efforts and expense in the acquisition of additional germplasm. Special effort should be made to preserve germplasm of known value. The overall research, labor, and money currently devoted to the preservation of field and forage crop germplasm is totally inadequate in the opinion of the writer. One outstanding example is that of the incomplete and inadequate maintenance of tropical and subtropical forage species. A location with suitable climate is necessary for the sustained maintenance of this germplasm whether by seed or vegetative propagation, especially the latter. Unfortunately, provision has not been made for such a site in the continental USA. Possible sites for the location of maintenance nurseries are Hawaii and Puerto Rico. Past experience indicates that such maintenance responsibilities should be specifically assigned to an appropriate agency, usually of the USDA.

It is not the task and responsibility of individual states or private institutions to conserve field and forage crop germplasm. This responsibility lies mainly with the USDA through an appropriate agency. Germplasm Resources Laboratory is a focal point within the USDA in the acquisition, distribution, and preservation of field and forage crop germplasm. It is the responsibility of this laboratory, along with that of Regional Plant Introduction Stations and the National Seed Storage Laboratory, to preserve this valuable germplasm. Sufficient and appropriate funding should be provided to accomplish this necessary continuous objective in its entirety. More specifically, provision should be made for the permanent maintenance of tropical and subtropical field and forage crop germplasm in the form of seed and vegetative propagating stocks.

The working seed stocks of the USDA World Collection of rice germplasm are maintained in Building 046, ARC West, in Beltsville. The current collection consists of approximately 16,000 entries. Further characterization of the collection consists of (1) plant introductions, (2) special genetic stocks, including trisomics, (3) foreign cultivars and land races, and (4) domestic released cultivars. Germplasm identified as to specific disease and insect resistance and salt tolerance is also included in the collection. Additional quantities of these seed stocks are maintained at NSSL under long-term storage and as insurance against possible loss at Beltsville. Inasmuch as rice is a quarantine crop, all raw germplasm is grown in quarantine. Currently, this is being accomplished by greenhouse plantings at Beltsville and in field plantings at Imperial Valley Field Station, El Centro, California. All working seed stocks are increased through the cooperation of rice research stations located in Arkansas, California, Louisiana, and Texas. The reciprocal flow of rice germplasm in and out of the United States is channeled through this Laboratory.

In view of the continuity necessary in the sustained preservation of rice germplasm and the predicted increase in volume of the collection, the following problems should be addressed now and within the foreseeable future. Paramount among these is that of additional storage space at Beltsville which requires immediate attention. The space allocated to growing rice in quarantine in the greenhouse at Beltsville is limited. This, coupled with the time required to produce a seed crop, usually of inadequate quantity, render it infeasible to grow large quantities of rice introductions under these conditions. This problem may be largely solved if extensive increase of rice germplasm at El Centro is successful and if the cooperative effort can be extended on a long-term basis. Our supply of working stocks, renewable through field increase, is contingent upon the continued cooperation of rice research stations. Continued research is required in long-term storage problems associated with the preservation of rice germplasm; the location of this research would be NSSL, Fort Collins, Colorado.

Collections of Microorganisms with Reference to Cooperative Research on a National Collection of Plant Viruses and Antisera

R. H. Lawson, Ornamentals Laboratory, PGGI

H. Hsu, American Type Culture Collection

Culture collections of microorganisms vary in size from small holdings to large national and international collections of fungi, bacteria, protozoa, mycoplasmas, plant and animal viruses and nematodes. Some smaller collections have well documented and authenticated strains of microorganisms that can be considered type cultures, but many others do not have this documentation. Larger collections often include more diverse holdings of value to both animal and plant science. These repositories are a source of authentic type cultures for distribution to the scientific community for identification and comparative taxonomy, as well as for many industrial uses. Collections in this category are in England, Holland, Japan and the United States. In this country large collections are in some instances maintained in government laboratories, but the largest collection of authentic type cultures is maintained at the American Type Culture Collection (ATCC) in Rockville, Maryland. ARS is currently surveying the status of collections of microorganisms as a first step in developing a national plan to encourage conservation, development and maintenance of type collections of microorganisms.

A collection of plant viruses was initiated nearly 20 years ago at the ATCC. The collection grew slowly and not until 1975 was adequate funding available to employ a professional plant virologist to oversee the collection. The program now provides ARS virologists with type cultures from a collection of more than 200 viruses.

Under a cooperative agreement with ARS, new and improved methods of storing plant viruses in tissue and in purified form are being developed. Virus type cultures are purified and antigens prepared for antiserum production at the ATCC. ARS provides electron microscope capability to assess the purity of antigens and study the structures of viruses and virus induced inclusions *in vitro* and *in situ*. Highly purified antigens have been produced to tomato ringspot, cucumber mosaic and carnation mottle viruses during the past year and antisera are being prepared. Plans have been developed to expand the production of antisera to develop a national antiserum bank. This bank will be useful to ARS to establish the identity of unknown viruses from virologists in the field. Early detection and positive identification of a virus may help limit distribution and control local outbreaks. Identification of unknown viruses would also give plant breeders the opportunity to search for sources of resistant germplasm before a disease is widespread.

The World Collection of sugarcane germplasm is currently maintained vegetatively by ARS in Beltsville and at Canal Point, Florida. This collection contains several thousand clones and it requires lots of labor and other expenditures for maintenance. It would be worthwhile if a more economical method for preservation could be found. Sugarcane can be cultured *in vitro* and much is known about the system. The method of preservation which would seem most interesting is liquid nitrogen storage of cells and tissues in cultures because of the long period of storage possible. Nag & Street (1975) preserved cell cultures of carrot, belladonna, and sycamore for up to 10 months with no changes in cell morphology or embryogenic potential. They froze cell cultures in DMSO and glycerol at a rate of 1-2° C/min in a type R 201 G.V. Planer Ltd., U.K. programmed freezer to 100° C and then into LN_2 for storage. Thawing was in a 37° C water bath. (Viability was assessed by fluorescein diacetate staining.)

SEIBERT (1976) REPORTED FREEZING SHOOT APICES OF CARNATION IN LIQUID NITROGEN FOR 2 MONTHS. DMSO WAS INCORPORATED AS A CRYOPROTECTANT. THE APICES WERE FROZEN RAPIDLY AT ABOUT 10000 C/MIN IN LIQUID NITROGEN AND WERE THAWED IN A 37° C WATER BATH. NORMAL PLANTS WERE PRODUCED FROM THESE APICES.

THE QUESTIONS FOR SUGARCANE GERMPLASM WOULD BE AS WITH ANY OTHER. FIRST, WILL IT SURVIVE THIS TYPE OF STORAGE, AND SECOND, EVEN IF IT SURVIVES AND PRODUCES PLANTS, WILL THE STORAGE ALTER THE GENETIC MAKE-UP OF THE SUGARCANE CLONES?

A SECOND AND SOMEWHAT DIFFERENT PROBLEM FOR US DEALS WITH SWEET SORGHUM POLLEN. ALL OF THE BREEDING WORK FOR SWEET SORGHUM IN ARS IS DONE AT BELTSVILLE IN THE GREENHOUSE. IT HAS BEEN DIFFICULT TO COORDINATE THE FLOWERING OF MALE AND FEMALE PARENTS AND IT IS DIFFICULT TO OBTAIN SATISFACTORY POLLEN AT SOME TIMES. THUS, A METHOD FOR PRESERVATION OF POLLEN WOULD BE ADVANTAGEOUS. POLLEN HAS BEEN STORED BY VACUUM-DRYING, FREEZING-DRYING, FREEZING, COOLING BUT NOT FREEZING, AND ORGANIC SOLVENTS. THERE ARE CONFLICTING REPORTS ON WHICH METHODS WORK FOR GRASS POLLENS. ALSO, THE METHODS FOR DETERMINING VIABILITY CONFLICT.

Nath & Anderson (1975) reported preservation of corn pollen for 25 days by rapid freezing (200° C/min) and rapid thawing (218° C/min) and for 180 days by rapid freezing, drying at -60° C for 75 hrs, storing in vacuum or nitrogen at -196° C, rehydrating at 5° C at 10% relative humidity. Viability was judged on a germinating medium. King (1961) had no luck in freeze-drying corn pollen, but he reported 140-day storage of freeze-dried sugarcane pollen, stored in nitrogen at room temperature. Viability was judged by peroxidase test.

Moore (1976) reported that sugarcane pollen did not store well by freeze-drying or freezing. He used a germination medium and said that the peroxidase test and pollination tests give false unreliable data. He stored sugarcane pollen for 14 days at 2° C at 100% relative humidity.

Nitzsche (1970) stored ryegrass pollen for 39 days at -20° C in a mixture of powdered polyvinylpyrrolidone and thawed at room temperature for 15 minutes. Viability was tested on a male sterile ryegrass clone.

For sweet sorghum pollen, we are working on developing an artificial germinating medium in addition to testing on a male sterile clone. We have tested media containing sucrose and some also containing calcium nitrate, boric acid, magnesium sulfate, and potassium nitrate. So far we have not found one adequate for our pollen. Freeze-drying, freezing, and storage at 2-4° C seem to be the methods to test for sweet sorghum.

Preservation of Sugarcane and Sweet Sorghum Germplasm

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We are presently attempting to work out a medium and the conditions for germinating pollen of sweet sorghum. As soon as this is accomplished, we will investigate methods for storage of this pollen as an aid for the breeding program. Storage at a temperature just above freezing under high humidity will be tested as well as the cryogenic techniques.

As a result of the workshop, cooperative preliminary experiments with the cryogenic storage of sugarcane cell and tissue cultures have been planned with Dr. Schaeffer of the Tissue Culture Lab. Additional experimentation in this area depends on the results of the preliminary tests and on whether or not equipment can be acquired at ARC for controlling the rate of freezing and thawing.